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Autonomous detection of aerosolized biological agents by multiplexed immunoassay with PCR confirmation

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Abstract

The autonomous pathogen detection system (APDS) is an automated, podium-sized instrument that continuously monitors the air for biological threat agents (bacteria, viruses, and toxins). The system has been developed to warn of a biological attack in critical or high-traffic facilities and at special events. The APDS performs continuous aerosol collection, sample preparation, and detection using multiplexed immunoassay followed by confirmatory PCR using real-time TaqMan assays. We have integrated completely reusable flow-through devices that perform DNA extraction and PCR amplification. The fully integrated system was challenged with aerosolized *Bacillus anthracis*, *Yersinia pestis*, *Bacillus globigii* and botulinum toxoid. By coupling highly selective antibody and DNA based assays, the probability of an APDS reporting a false positive is extremely low.

INTRODUCTION

Aerosol monitoring systems that test for the presence of biological agents are in use throughout the United States.[1] Their purpose is to detect a biological agent release so that early public health action may be taken. The impact of a biological agent attack can be reduced if the appropriate responses are executed with minimal delay.[1] For example, at the 2002 Winter Olympics in Salt Lake City, aerosol samples from dry filter collectors were periodically transported to a centralized field laboratory where they were screened for the presence of pathogen specific nucleic acid sequences.

The autonomous pathogen detection system (APDS) is a next-generation biological agent detector that was developed to realize in-situ sample collection, analysis and result reporting. The benefits of this approach include reduced operational costs and faster data reporting whilst maintaining a low false positive rate. McBride et al[2] described an earlier version of APDS that performed aerosol collection, sample preparation, and multiplexed immunoassay detection; detection of live *Bacillus anthracis* and *Yersinia pestis* was demonstrated during laboratory chamber testing of the device. Since this report, DNA detection capability has been added to the APDS to further reduce the likelihood of false positives; now, a suspect immunoassay result initiates a confirmatory real time flow-through PCR assay. Belgrader et al[3] have described the real-time flow-through PCR module employed in APDS. As certain sample matrices can interfere with the PCR assay, a DNA extraction device was also added.

In its current configuration, the APDS operates continuously for 7 days; an immunoassay result is reported every hour and a control PCR reaction is executed twice daily. A suspicious immunoassay result is identified using a signal analysis algorithm, which triggers the corresponding confirmatory PCR assay. Together, these technical advances culminated in laboratory chamber tests during which the APDS was challenged with individual aerosol releases of *B. anthracis*, *Y. pestis*, *B. globigii* (a *B. anthracis* simulant) and botulinum toxoid (inactivated botulinum toxin). The APDS data presented herein demonstrates for the first time completely autonomous biological monitoring using aerosol sample collection, multiplexed immunoassay analysis, DNA extraction, and confirmatory PCR.

EXPERIMENTAL

The previous version of the APDS, which incorporated an aerosol collector, a sample preparation module and multiplexed immunoassay flow cytometric detection, has been described in previous papers.[2, 4] The current APDS also employed a Luminex 100 flow cytometer (Luminex Corp., Austin, TX) fitted with a sheath delivery system (Luminex). Antibodies from Tetracore (Gaithersburg, MD) were conjugated to Luminex microspheres (Luminex) according to the manufacturers protocols. The automated fluidics was a modified sequential injection analysis system (Global FIA, Fox Island, WA) fitted with two syringe pumps (1mL, XP3000, Cavro, Sunnyvale, CA) and four multi-position selection Cheminert valves (2 × 10 and 2 × 14 port, VICI, Houston, TX). PFA tubing (0.8 mm ID, 1.6 mm OD, Cole-palmer, Vernon Hills, Illinois, USA) was used throughout the fluidics manifold. Flangeless ¼-28 and 10-32 nuts and ferrules (VICI) provided tubing connections throughout the manifold.

System Control. APDS components, including the aerosol collector, sample preparation module and Luminex 100 were controlled by a laptop computer running a graphical user interface written in Labview Version 6.1 (National Instruments, Austin, TX). Communication between the Labview graphical user interface and the Luminex 100 was achieved using the software program Luminex LXR Library Version 2.6.9 (Luminex). Both serial cables and data acquisition cards (DAQ1200, National Instruments) were used for hardware communications and data transfer throughout the system.

Immunoassay signal analysis. Baseline MFI values for 11 agents and 4 controls were established from samples (n=24) of blank chamber air that were collected and analyzed by the APDS at Dugway. For each microsphere class, the mean baseline value and standard deviation were calculated and entered into the system. During autonomous operation, the immunoassay results for given sample were first divided by their respective baseline MFI value to create a normalized signal. The highest agent normalized signal was then divided by the second highest normalized signal (including the remaining 10 agents and the negative control) to yield a relative signal, which accounted for baseline drift and non-specific binding. The relative signal was checked against an established threshold value calculated

from titration data collected previously in our laboratory. The system also checked that >1500 classified microspheres were counted and that control MFI values were valid (baseline MFI ±3σ). If a relative signal for a given agent exceeded its threshold, the corresponding confirmatory PCR reaction was triggered.

Automated DNA extraction. Christel et al[5] have described a micro-machined silica pillar device similar to that used within the APDS. The chip was fabricated within our laboratory using deep reactive ion etching to yield a square pillar bed with a void volume of 3 μL and a total surface area of ~2 cm^2. The pillar diameter, length, and spacing were 20 μm, 200 μm and 20 μm, respectively. A platinum thin film resistor was deposited on the backside of the chip to enable heating of the device during DNA elution.

Aerosol collector sample (100 μL of a total 4 mL) and chaotropic solution (100 μL, 2 *M* guanidine thiocyanate, 3.3 m*M* Tris, 0.33 m*M* EDTA, pH 6.5, Teknova, Hollister, CA) were mixed by the automated fluidics system then pumped through the silica bed at 1 µL/s to extract the DNA. The bed was washed with ethanol (300 μL of 70 % v/v, then 100 μL of 95 % v/v) to remove potential PCR interferents. The DNA was eluted in Tris/EDTA buffer (10 μL, 10 m*M* Tris, 1 mM EDTA, pH 8.0, Acros, Morris Plains, NJ) with heating to 80°C, and then introduced as sample (5 μL) to the flow through PCR module. After each extraction the chip was decontaminated with sodium hypochlorite (100 µL, 1.25% m/v) then rinsed with deionized water (2 mL).

Automated flow-through PCR. The flow-through PCR module has been described previously.[3] Within the APDS, the automated fluidics module assembled each PCR reaction (25 µl) from enzyme master mix solution (15 µL, Accuprime Supermix I, $MgCl_2$ 4 m*M*, Invitrogen, Carlsbad CA), a mixed solution of DNA primer pairs and TaqMan probe (5 µL, 200 n*M* each, probe was FAM at 5' end quenched by Black Hole Quenchers at 3' end, Biosearch Technologies, Novato CA) and sample (5 µL). The resultant reaction mixture was positioned in the tubing of the flow-through PCR heater and

subjected to a thermal cycling protocol of 95ºC for 120 s followed by 40 cycles of 95ºC for 15 s, 60ºC for 30 s, 72ºC for 15 s. A fluorescence measurement (480 nm excitation, 520 nm emission) was made during each cycle, after completion of the 60ºC hold. After thermal cycling had finished, the reactor tubing was automatically cleaned with sodium hypochlorite (500 μL, 1.25 % m/v) followed by a water rinse (4 mL). This cleaning procedure prevented any carryover of amplified DNA and regenerated the PCR module for the next assay.

Biological agents. Certified killed (gamma-irradiated) *B. anthracis* spores (Ames strain) and *Y. pestis* vegetative cells (India 195/p strain) were from Dugway Proving Grounds (Dugway, UT). Viable *B. globigii* spores were also from Dugway Proving Grounds. Botulinum toxoid (an inactivated form of botulinum toxin) was from Tetracore.

Aerosol Chamber Test Procedure. The APDS was tested in the Aerosol Simulant Exposure Chamber at the West Desert Test Center, United States Army Dugway Proving Grounds. The chamber (5 × 5 × 3 m) was equipped with a Babbington aerosol generator (referred to here as the nebulizer) that aerosolized liquid samples at variable flow rates (0.2 to 5 mL/min). The subsequent aerosol was thoroughly mixed with chamber air by a bank of fans; each release lasted for 50 min, and coincided with the collection period. The air turn over rate within the chamber was between 1400-14000 L/min. The aerosol collector was removed from the chassis and positioned in the center of the chamber whilst other APDS components remained outside the chamber; fluid and communication connections were made via a sealed port. The liquid sample was automatically pumped from the aerosol collector to the fluidics module hourly.

Bioaerosol reference measurements were made. An aerodynamic particle sizer (APS) provided real-time particle size measurements (0.5 to 30 μm) prior to and during each release. All-glass impinger air samplers (AGI-30s, Ace Glass Co. Vineland, NJ) and slit-to-agar biological air samplers (New Brunswick Scientific, Edison, NJ) were also utilized. For disseminations, antigen slurry (100 mL, prepared in deionized water) was loaded into the nebulizer. A target APS count was maintained for the duration of each release by cycling on and off the nebulizer's peristaltic pump.

Decontamination. After each agent release, the chamber was purged with clean air, until the APS indicated that the particle counts had returned to baseline. The nebulizer, AGIs, and slit samplers (when used) were retrieved from the chamber. Both the chamber floor and aerosol collector were decontaminated with sodium hypochlorite (0.6% m/v) then rinsed with water. The water from the final aerosol collector rinse was collected then analyzed by real-time PCR to verify this component was clean before we proceeded with the next test.

Safety Considerations. Personal protective equipment (gloves, lab coat, goggles, positive air pressure respirators) was worn. Consumables (filtration plates, pipette tips, tubes etc.) and aqueous waste were collected in biohazard bags and autoclaved. The instrumentation was decontaminated with sodium hypochlorite solution (1.25% m/v) after exposure to biological agents.

RESULTS AND DISCUSSION

The Autonomous Pathogen Detection System (APDS). A process flow diagram of the APDS with PCR confirmation is shown in Scheme 1. The aerosol collector samples up to 3300 liters of air/min, and airborne particles are trapped in a small volume of water (4 ml) within a wetted wall cyclone that is analyzed periodically (hourly in this case). The multiplex immunoassay is the primary screen and the instrument operates in the first loop (Scheme 1, left hand side) for most of the time. A reactive immunoassay result causes a Level 1 response that represents actions taken by both the instrument (e.g., paging an expert with a request for data review) and by external systems (e.g., security cameras checked). If the reactive agent contains DNA, then PCR confirmation is initiated. A reactive PCR assay result causes a more extensive Level 2 response. The use of two highly specific biological assays, that each rely on fundamentally different molecular interactions for detection, boosts confidence in the results, which in turn enables decisive (Level 2) responses to begin before samples could even be transported to a field laboratory for analysis.

Multiplexed Microsphere Immunoassay. The current multiplexed microsphere immunoassay is an extension of those described previously.[2, 4, 6] In short, antigens form a "sandwich" with antibody-coated microspheres (5.6 μm) and detector antibodies labeled with streptavidin-phycoerythrin; the resulting suspension is read with a Luminex 100 flow cytometer. The microspheres are color-coded to identify the antigen type; the fluorescence intensity of bound streptavidin phycoerythrin corresponds to antigen concentration. Up to 100 different microsphere classes can be distinguished in one sample. The assay panel used in this study tested for eleven biological agents simultaneously, including *B. anthracis*, *Y. pestis*, *B. globigii* (a *B. anthracis* simulant), and botulinum toxoid (inactivated botulinum toxin). In addition to the eleven agent microspheres types, four control microsphere classes were included in each assay: negative control, instrument control, fluorescent control, and antibody control.[2, 4] These internal assay controls provide important diagnostic information during extended autonomous operation.

Polymerase chain reaction (PCR) assays. The APDS uses DNA assays based on real-time TaqMan PCR, where cleavage of fluorescent resonance energy transfer (FRET) quenched probes during amplification results in an increased fluorescence signal.[7, 8] The assay is performed in a flow-through PCR module described by Belgrader et al.[3] We took this module and made the PCR analysis fully automated, i.e. after a suspicious immunoassay result the system selected the corresponding PCR reagents, mixed them with sample, performed the PCR analysis, and then decontaminated fluid lines and components in preparation for the next analysis. During routine operation, the system can accommodate parallel execution of both the immunoassay and PCR.

Nucleic acid extraction. Certain environmental sample matrices contain species that can inhibit PCR and prevent DNA amplification from occurring. In order to purify nucleic acids from inhibitors that could be collected from the environment, the APDS performs sample preparation by microchip based solid-phase extraction prior to PCR. In this process DNA is captured on micro fabricated silica pillars, while the sample matrix including potential interferents pass through to waste. The immobilized DNA is washed, eluted, and then introduced into the PCR reaction. During our testing at Dugway, the APDS collected samples of relatively clean chamber air that did not contain PCR inhibitors. The nucleic acid extraction module, however, was enabled during chamber tests to evaluate its function as an integral component of the APDS. Input and elution volumes used by the extraction module were 100 and 10 µl respectively. For the extraction module alone, input *B. anthracis* DNA concentrations ranging from 10^3 to 10^7 copies/mL, concentrations factors of ~1-2 or recoveries of ~10-20% were obtained (data not shown). As PCR is at least 50 fold more sensitive than our multiplexed immunoassay, this extraction performance was adequate for the confirmatory PCR assay. Automated microchip DNA purification from PCR inhibitory environmental aerosol samples will be published in a separate report.

Chamber testing of the APDS with aerosolized biological agents. We conducted releases of *B. anthracis* spores, *Y. Pestis* vegetative cells, *B. globigii* spores and botulinum toxoid. Each test point consisted of collecting the disseminated aerosol, preparing and reading the multiplexed immunoassay,

identifying a reactive immunoassay result with a signal analysis algorithm, DNA extraction, real-time flow-through PCR, and observing a cycle threshold, all in an unattended continuous mode of operation. Confirmatory PCR was not performed for the toxoid release; being a protein the presence of target DNA was not guaranteed. For these experiments, the system ran autonomously overnight and collected hourly aerosol samples of clean chamber air (blanks) that were analyzed by multiplexed immunoassay; an agent was released the following morning without interrupting the APDS. After the system had completed its analysis of the release, it was stopped, and then decontaminated.

The multiplexed immunoassay results for *B. anthracis* are shown in Figure 1, where median fluorescence intensities (MFI) of the 11 agents and the negative control are plotted versus time. The MFI values for all microsphere classes remained within 6σ of their mean baseline value until a sudden increase of the *B. anthracis* signal that coincided with the aerosolization of *B. anthracis* spores. Note that the MFI values of the other microsphere classes remained within 6σ of their mean baseline in the presence of *B. anthracis*, indicating our multiplexed immunoassays are highly specific. The subsequent PCR assay was triggered and provided clear confirmation of the reactive immunoassay result. We used deionized water as negative control for the PCR confirmation assay prior to the *B. anthracis* release, which verified the system was clean prior to the release.

The initial *Y. Pestis* release (Figure 2) generated an MFI value that was not detected by the signal analysis algorithm in use at the time and as a result, confirmatory PCR was not initiated. The *Y. pestis* aerosol concentration was increased during the next collection cycle; the increased MFI value in the subsequent immunoassay was detected by the algorithm and triggered confirmatory PCR with an unequivocal result. We have since changed the signal analysis algorithm used by the APDS to a moving window average based upon partial least squares regression.

The APDS data from a *B. globigii* release is also shown in Figure 3. We used viable *B. globigii* spores to estimate the number of colony forming units (c.f.u.) present in the air during this dissemination. Slit samplers collected airborne particles directly onto culture plates that were subsequently incubated then analyzed. Using this method, we calculated that the average agent-

containing-particle-per-liter-of-air (ACPLA) value was 49. The results from this experiment demonstrate that the APDS is capable of achieving the sensitivity required by many real world environmental monitoring scenarios.

Finally, detection of aerosolized botulinum toxoid is shown (Figure 4), which highlighted the versatility of the multiplexed immunoassay platform in its ability to identify the presence of pathogenic proteins, in addition to bacteria.

CONCLUSIONS

We have demonstrated, for the first time, completely autonomous detection of aerosolized *B. anthracis* and *Y. Pestis* by multiplexed immunoassay with confirmatory PCR. With the addition of DNA detection capability, the probability of an APDS reporting a false positive is extremely low. We also realized the detection of a botulinum toxoid aerosol that highlighted the system's ability to respond to protein toxins, in addition to bacteria. We have since have fabricated multiple APDS units, equipped with the functionality described and demonstrated herein, that are undergoing extensive environmental testing in real-world environments, including subways and airports. The nucleic acid extraction module has undergone further characterization; the automated purification of DNA from environmental aerosol samples known to inhibit the PCR reaction will be described in a separate report. The next version of the APDS, to be field tested this year, will incorporate multiplexed PCR with Luminex microsphere-based hybridization detection.

ACKNOWLEDGMENTS

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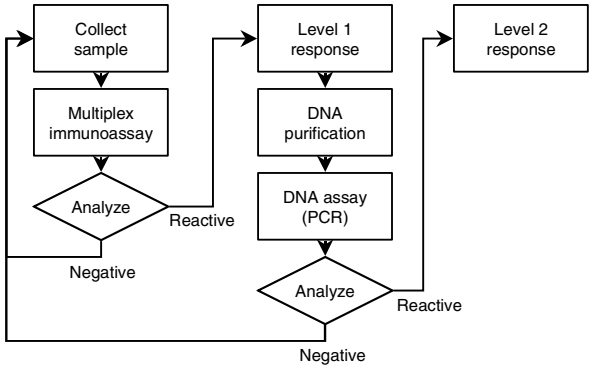

Scheme 1. APDS flow diagram showing the cycle of sample collection, multiplexed immunoassay, DNA extraction and confirmatory PCR analysis. Assay results and system status are continuously transmitted through a secure wireless network.

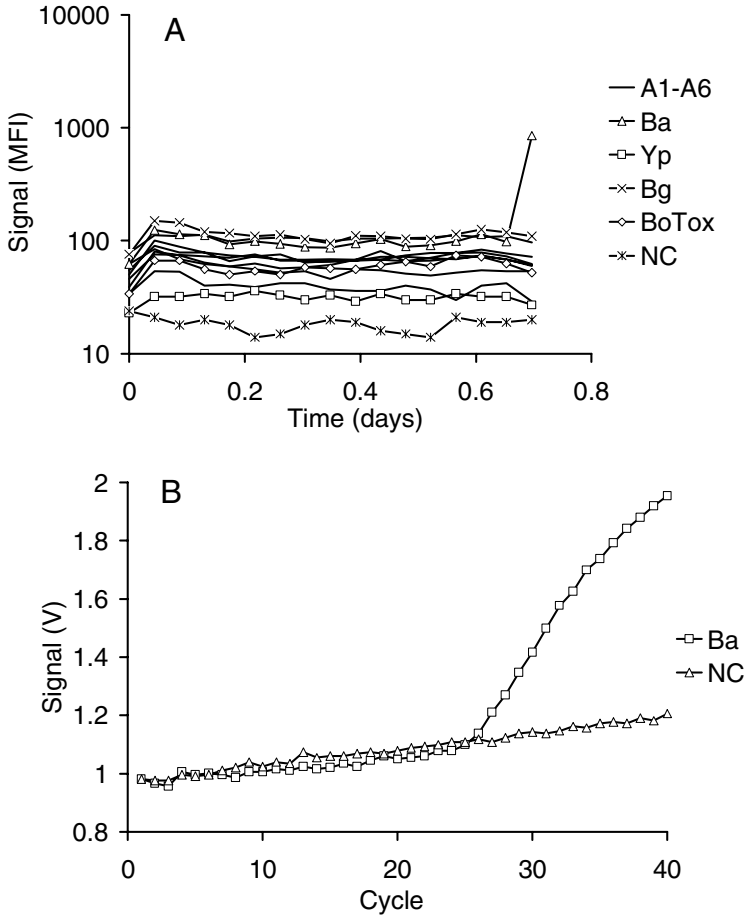

Figure 1. Autonomous pathogen detection system chamber test results that demonstrated (A) multiplexed immunoassay identification followed by (B) DNA extraction with PCR confirmation of an individual aerosol release of *B. anthracis*. Immunoassay signal designations are A1-A6 (Agents 1-6), Ba (*B. anthracis*), Yp (*Y. pestis*), Bg (*B. globigii*), BoTox (Botulinum toxoid) and NC (negative control). MFI is the median fluorescence intensity shown for 11 agents and the negative control. Chamber background aerosol samples were collected and analyzed by the system prior to each release. A negative control (NC) for PCR confirmation is shown for reference; this was obtained prior to the *B. anthracis* release (A) using deionized water as the sample.

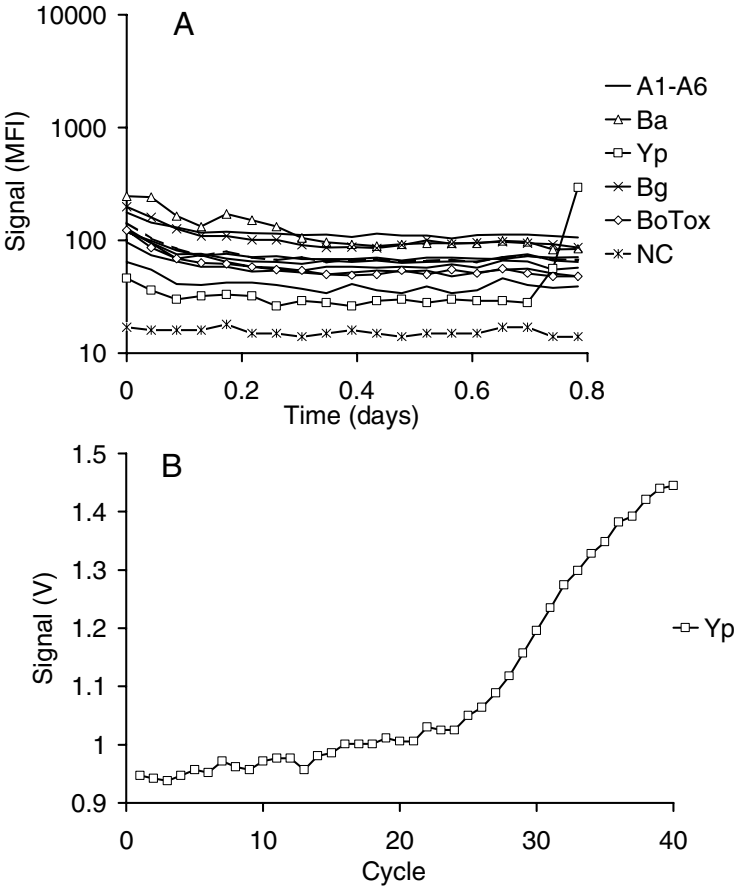

Figure 2. Autonomous pathogen detection system chamber test results that demonstrated (A) multiplexed immunoassay identification followed by (B) DNA extraction with PCR confirmation of an individual aerosol release of *Y. pestis*. Immunoassay signal designations are A1-A6 (Agents 1-6), Ba (*B. anthracis*), Yp (*Y. pestis*), Bg (*B. globigii*), BoTox (Botulinum toxoid) and NC (negative control). MFI is the median fluorescence intensity shown for 11 agents and the negative control. Chamber background aerosol samples were collected and analyzed by the system prior to each release.

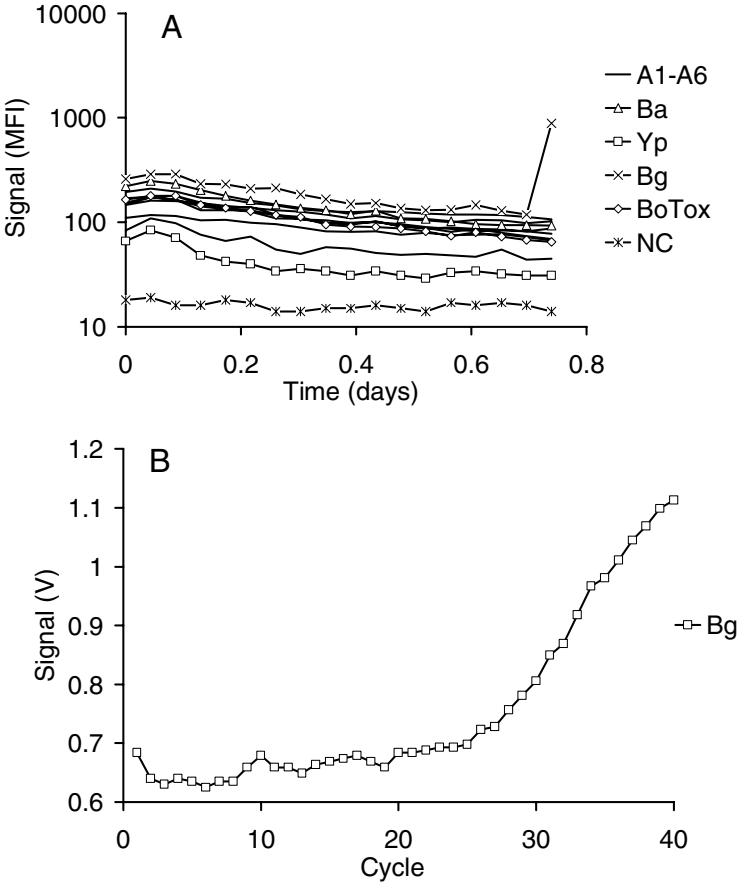

Figure 3. Autonomous pathogen detection system chamber test results that demonstrated (A) multiplexed immunoassay identification followed by (B) DNA extraction with PCR confirmation of an individual aerosol release of *B. globigii*. Immunoassay signal designations are A1-A6 (Agents 1-6), Ba (*B. anthracis*), Yp (*Y. pestis*), Bg (*B. globigii*), BoTox (Botulinum toxoid) and NC (negative control). MFI is the median fluorescence intensity shown for 11 agents and the negative control. Chamber background aerosol samples were collected and analyzed by the system prior to each release.

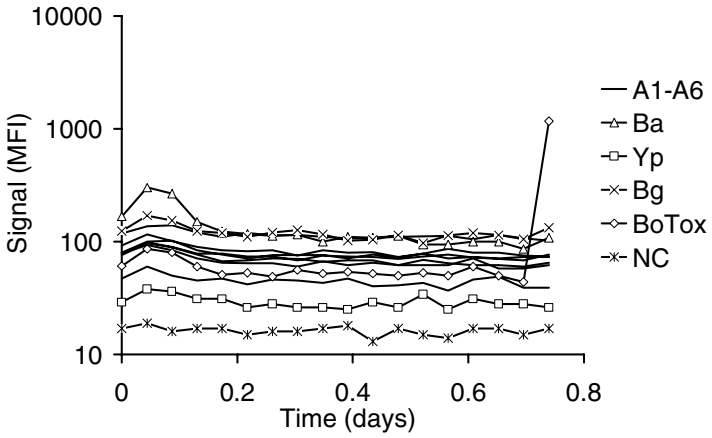

Figure 4. Autonomous pathogen detection system chamber test results that demonstrated multiplexed immunoassay identification of an individual aerosol release of Botulinum toxoid. Immunoassay signal designations are A1-A6 (Agents 1-6), Ba (*B. anthracis*), Yp (*Y. pestis*), Bg (*B. globigii*), BoTox (Botulinum toxoid) and NC (negative control). MFI is the median fluorescence intensity shown for 11 agents and the negative control. Chamber background aerosol samples were collected and analyzed by the system prior to each release.

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